The Effects of Mitral Regurgitation Alone Are Sufficient for Leaflet Remodeling

Elizabeth H. Stephens, BS; Tom C. Nguyen, MD; Akinobu Itoh, MD; Neil B. Ingels, Jr, PhD; D. Craig Miller, MD; K. Jane Grande-Allen, PhD

Background—Although chronic mitral regurgitation results in adverse left ventricular remodeling, its effect on the mitral valve leaflets per se is unknown. In a chronic ovine model, we tested whether isolated mitral regurgitation alone was sufficient to remodel the anterior mitral leaflet.

Methods and Results—Twenty-nine sheep were randomized to either control (CTRL, n=11) or experimental (HOLE, n=18) groups. In HOLE, a 2.8- to 4.8-mm diameter hole was punched in the middle scallop of the posterior mitral leaflet to create “pure” mitral regurgitation. At 12 weeks, the anterior mitral leaflet was analyzed immunohistochemically to assess markers of collagen and elastin synthesis as well as matrix metalloproteinases and proteoglycans. A semiquantitative grading scale for characteristics such as intensity and delineation of stain between layers was used to quantify differences between HOLE and CTRL specimens across the heterogeneous leaflet structure. At 12 weeks, mitral regurgitation grade was greater in HOLE versus CTRL (3.0±0.8 versus 0.4±0.4, P<0.001). In HOLE anterior mitral leaflet, saffron-staining collagen (Movat) decreased, consistent with an increase in matrix metalloproteases throughout the leaflet. Type III collagen expression was increased in the midleaflet and free edge and expression of prolyl-4-hydroxylase (indicating collagen synthesis) was increased in the spongiosa layer. The proteoglycan decorin, also involved in collagen fibrillogenesis, was increased compared with CTRL (all P≤0.05).

Conclusions—In HOLE anterior mitral leaflet, the increased expression of proteins related to collagen synthesis and matrix degradation suggests active matrix turnover. These are the first observations showing that regurgitation alone can stimulate mitral leaflet remodeling. Such leaflet remodeling needs to be considered in reparative surgical techniques.

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Key Words: collagen ■ immunohistochemistry ■ regurgitation ■ remodeling ■ valves

Mitral regurgitation (MR) is associated with a number of chronic heart diseases. The prevalence of at least mild severity MR is reported to be approximately 19%,1 and MR in the context of dilated cardiomyopathy (DCM) portends a significantly worse prognosis.2–4 It has been hypothesized that the MR in DCM is “functional,” ie, primarily due to left ventricular enlargement and annular dilatation resulting in impaired leaflet coaptation.5–7 Recent reports of altered leaflet matrix composition in patients with heart failure,8 however, suggest that the leaflet itself is involved in the changes associated with heart failure, perhaps contributing to MR. Indeed, the severity of MR in DCM often increases over time, a phenomenon referred to as “MR begets MR.”9 However, given that many factors contribute to MR in DCM, including ventricular and annular remodeling, ischemic damage, and low-pressure volume overload,9,10 it is unclear which variables drive the worsening regurgitation. The hypothesis of this study was that the altered hemodynamics of regurgitation alone could lead to adverse leaflet remodeling, thereby providing a possible mechanism for “MR begets MR.” The goal of this study was to investigate the effect of the isolated hemodynamic variable by creating “pure” primary MR (independent of left ventricular dysfunction) and examine the anterior leaflet for signs of adverse remodeling. The implications of this study would be that the mitral valve during the course of chronic MR could contribute to worsening MR. Conversely, this causal relationship would also imply that improvements in valve hemodynamics after mitral valve repair surgery could reverse negative remodeling of the valvular extracellular matrix (ECM).

To test this hypothesis, an ovine animal model of “pure” regurgitation was created by punching a 2.8- to 4.8-mm hole in the center of the posterior leaflet of the mitral valve. MR was monitored for 12 weeks before the animals were euthanized and the anterior mitral leaflet (AML) was analyzed to determine if adverse remodeling had occurred. Remodeling

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was assessed by histological analysis of ECM constituents as well as markers of matrix turnover. ECM not only serves as a structural scaffold, but also plays an active role in critical processes such as tissue differentiation, cell migration, growth factor regulation, mechanical function, and disease in various tissues, including heart valves. In addition, given reports of cell activation and proliferation at the site of valve injury, these characteristics were examined as well.

**Materials and Methods**

All animals received humane care in accordance with the guidelines of the US Department of Health and Human Services (National Institutes of Health Publ 85-23, Revised 1985). The use of animals in this study was approved by the Stanford Medical Center Laboratory Animal Research Committee.

**Surgical Protocol**

Twenty-nine sheep were randomized to either control (CTRL, n = 11) or experimental (HOLE, n = 18) groups. Epicardial echocardiography was used to qualitatively grade (0 to 4) MR at baseline based on color and width of the regurgitant jet. A left thoracotomy and atriotomy were used to access the mitral valve, and after establishment of cardiopulmonary bypass, a 2.8-mm to 4.8-mm hole was created in the central scallop of the posterior mitral leaflet of HOLE animals with an aortic hole puncher (Figure 1). CTRL animals underwent the exact same operation without the hole punch. On a weekly basis, a blinded echocardiographer performed transthoracic echocardiography and graded the MR on the basis of color Doppler regurgitant jet extent and width. The MR grades taken at 6 and 12 weeks were combined to determine the “averaged final MR.” The end diastolic volume index and end systolic volume index were calculated based on the sum of 3-dimensional volumes enclosed by the mitral annular and left ventricular markers indexed to the body surface area. The full explanation of these calculations and the description of additional measurements performed using this model have been published previously.

**Histology and Immunohistochemistry**

The AML was isolated and a 5-mm wide strip was cut from the annulus to the free edge (Figure 2A). To control for heterogeneity in loading and microstructure among different regions of the AML, the strip was taken from the same region in all animals. These cross-sections were embedded in paraffin and sectioned to a thickness of 5 μm. A cross-section was also taken from each posterior leaflet (including the region with the hole punch) and subjected to similar studies as those performed here for the AML. Those results are reported in a separate article. Each sample was stained histologically with Movat pentachrome to demonstrate the general collagen (yellow), elastic fiber (black), and proteoglycan/glycosaminoglycan content (blue) and to distinguish between the different layers of the mitral valve. Picrosirius red staining was performed to examine the collagen content and alignment and infer type of collagen (red=collagen I, yellow–green=reticular collagen III). Samples were also stained immunohistochemically to demonstrate specific ECM components and enzymes involved in matrix turnover (Table), including the proteoglycan decorin (DCN), involved in collagen fibrillogenesis; Type III collagen (Col III), which tends to be unregulated in remodeling tissues; the elastic fiber-related proteins elastin, fibrillin, and lysyl oxidase (involved in crosslinking both collagen and elastin); 2 markers of collagen synthesis, prolyl 4-hydroxylase (P4H) and heat shock protein-47; and markers of matrix degradation including the matrix metalloproteases (MMPs)-1,-2,-9, and -13. To determine whether the regurgitation affected the phenotype of the cells within the valve, cell proliferation and valve cell activation were evaluated using antibodies against proliferating cell nuclear antigen and nonmuscle myosin, respectively. To limit variability due to staining procedures, all leaflets (both HOLE and CTRL) were stained for a given marker together in one batch. A semiquantitative grading scale from 0 to 4 was used to assess the intensity of staining and the delineation of stain between layers both throughout the leaflet and in the insertion, annulus, midleaflet, and free edge regions (Figure 3). In addition, “background elastin staining” was defined as the intensity of staining in the fibrosa and spongiosa layers, ie, between the strongly staining linear fibers found within the atrialis and ventricularis (see circled region in Figure 3B). All evaluations were performed by a trained individual blinded to the identity of the leaflets.

**Statistical Analysis**

Data are presented as mean and SD unless otherwise noted. Multifactorial analysis of variance was performed using SigmaStat (SPSS, Chicago, Ill). When the data were normally distributed, an analysis of variance test was used. When the data set was not normally
distributed, a rank transform was performed before the analysis of variance. In both cases, the level of significance was set at 0.05. In a few select cases, the probability value for a nonnormally distributed data set after rank transformation set was \( P < 0.05 \), whereas the original analysis of variance had a probability value \( P > 0.05 \). In these instances, the probability value from the original analysis of variance is listed followed by an "R." In all other cases of nonnormalized data sets, the probability value from the rank-transformed analysis of variance is listed. The Holm-Sidak all-pairwise multicomparison method was used for post hoc testing. Correlations between factors were calculated using a Spearman rank order correlation test with a significance level of 0.05.

**Statement of Responsibility**
The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

**Results**
Throughout the study, the MR grade was greater in HOLE than in CTRL (Figure 4). At 12 weeks, the grades were 0.4±0.4 versus 3.0±0.8 for CTRL versus HOLE, respectively \((P<0.001)\). The regurgitant jet was largely centrally oriented (toward the atrium; Figure 4B) and did not hit the AML directly. This MR was accompanied by a greater mitral annulus area, primarily due to an increase in the commissure-to-commissure dimension, although there was no evidence of change in the 3-dimensional annular shape. End diastolic

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**Table. Antibodies Used in Immunohistochemistry**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Function</th>
<th>Dilution</th>
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<tbody>
<tr>
<td>Collagen-related markers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCN*</td>
<td>PG associated with tension and collagen fiber formation</td>
<td>1:500</td>
</tr>
<tr>
<td>Col III†</td>
<td>Reticular type of collagen</td>
<td>1:100</td>
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<tr>
<td>P4H‡</td>
<td>Hydroxylating enzyme in collagen synthesis</td>
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<tr>
<td>Heat shock protein 47†</td>
<td>Molecular chaperone in collagen synthesis</td>
<td>1:200</td>
</tr>
<tr>
<td>Lysyl oxidase§</td>
<td>Crosslinking enzyme in collagen synthesis</td>
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<tr>
<td>Elastic fiber-related</td>
<td></td>
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<tr>
<td>Elastin†</td>
<td>Primary component of elastic fibers</td>
<td>1:150</td>
</tr>
<tr>
<td>Fibrillin†</td>
<td>Elastic fiber component</td>
<td>1:150</td>
</tr>
<tr>
<td>Lysyl oxidase§</td>
<td>Crosslinking enzyme in elastic fiber formation</td>
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<tr>
<td>Matrix degradation</td>
<td></td>
<td></td>
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<tr>
<td>MMP-1</td>
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<td></td>
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<tr>
<td>MMP-2</td>
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<tr>
<td>MMP-9</td>
<td></td>
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<tr>
<td>MMP-13‡</td>
<td>Enzyme involved in matrix degradation</td>
<td>1:200</td>
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<tr>
<td>Cell activation/proliferation</td>
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<td></td>
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<tr>
<td>Proliferating cell nuclear antigen†</td>
<td>Marker of cell proliferation</td>
<td>1:500</td>
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<tr>
<td>Nonmuscle myosin¶</td>
<td>Marker of an &quot;activated&quot; cellular phenotype</td>
<td>1:100</td>
</tr>
</tbody>
</table>

*Gift from Dr Larry Fisher, National Institutes of Health.
†Abcam, Cambridge, Mass.
‡Chemicon, Temecula, Calif.
§Imgenex, San Diego, Calif.
\(|\text{Assay Designs, Ann Arbor, Mich.}\)
¶Covance, Berkeley, Calif.
PG indicates proteoglycan.
Volume index and end systolic volume index were both significantly greater in HOLE compared with CTRL at 12 weeks (end diastolic volume index: $109.1 \pm 30.0$ mL/m² versus $146.3 \pm 30.3$ mL/m² for CTRL versus HOLE, respectively; end systolic volume index: $81.7 \pm 29.5$ mL/m² versus $106.3 \pm 18.7$ mL/m² for CTRL versus HOLE, respectively, $P<0.004$ for both). Levels of circulating biochemical markers such as blood natriuretic peptide were not significantly different between groups. The full details of cardiac changes observed in the HOLE animals have been published previously.15

Numerous differences in the staining of several ECM markers within the AML were found between HOLE and CTRL animals. Cell proliferation, as detected by proliferating cell nuclear antigen staining, tended to be greater in HOLE compared with CTRL ($P=0.089$), and nonmuscle myosin expression in the fibrosa positively correlated with the average final grade of MR ($r=0.225$, $P<0.027$). With respect to the overall amount of collagen, Movat-stained sections showed a significant reduction in saffron-staining collagen in HOLE ($P=0.041$; Figure 5). Picrosirius red staining confirmed that Movat saffron-stained regions were composed predominantly of Type I, rather than Type III, collagen. When viewed under polarized light, the large, linear bundles of Type I collagen fibers in picrosirius red-stained tissue appeared red, whereas the small-diameter, networked collagen Type III fibers appear yellow–green.17 HOLE AML showed greater Col III compared with CTRL ($P=0.024$; Figure 5), particularly in the midleaflet and free edge regions. HOLE AML also showed elevated P4H expression compared with CTRL ($P=0.024$), which was most notable in the spongiosa layer ($P=0.024$). DCN expression was greater in HOLE ($P=0.022R$), which also showed a trend of higher MMP-13 intensity compared with CTRL ($P=0.081R$). MMP-9 staining across all regions of HOLE AML was greater than in CTRL AML ($P=0.021$; Figure 6A); and MMP-1 staining in the fibrosa of the annulus and midleaflet regions was greater for HOLE compared with CTRL ($P=0.024$; Figure 6B). In these same 2 regions, the background elastin staining (ie, staining in the spongiosa and fibrosa) was significantly greater in the HOLE AML ($P=0.023$; Figure 7). In the insertion region of AML, there was greater staining for Col III in HOLE compared with CTRL ($P=0.048$) due to an abundance of Col III in the fibrosa layer ($P=0.002$). The HOLE AML insertion region also showed a trend of greater MMP-9 abundance ($P=0.070$) that was significant in the fibrosa layer ($P=0.022$).

**Discussion**

The principal findings of this study were that HOLE AML exhibited collagen remodeling demonstrated by a reduction in collagen Type I and elevated expression of Type III collagen, P4H, DCN, and MMP-1. Furthermore, HOLE AML showed evidence of elastic fiber remodeling, including greater MMP-9 and elastin expression within the fibrosa and spongiosa layers. Consistent with valvular matrix remodeling,
of “background” elastin may indicate altered mechanical loading of the AML. With respect to collagen remodeling, the stronger MMP-1 staining was specifically found in the fibrosa of the midleaflet and annulus, whereas the stronger Type III collagen staining was predominantly found in the midleaflet and free edge. Type I collagen is normally most abundant in the fibrosa layer within the annulus and midleaflet regions and is critical to the leaflet’s tensile strength. Given the specificity of MMP-1 for collagen Type I, it is logical that MMP-1 expression would be stronger in the fibrosa layer of these regions. It may be that the nature of the regurgitant jet and the altered coaptation patterns caused increased tension on the free edge and midleaflet regions, which could explain the greater abundance of Type III collagen in those regions. It is also interesting to note that the insertion region (annular ring) of HOLE AML showed greater abundance of Coll III and MMP-9.

We speculate that the MR resulting from the hole punch led to altered stress on HOLE AML, which led to altered collagen turnover throughout the various leaflet regions. Functionally, in the midleaflet region, this altered collagen content would reduce tensile strength, leading to leaflet prolapse that could worsen MR. Altered collagen turnover in the insertion region (part of the mitral fibrous annulus) would result in decreased tensile strength of this region and lead to annular dilation, as evident in HOLE. This annular dilation would also then worsen MR. Although less annular dilation occurs in the anterior as opposed to the posterior mitral annulus in DCM, anterior annular dilation has been documented in both animal models and human cases of DCM.

Potential Mechanism for Observed Changes

The proposed mechanism for the observed dysfunction in the HOLE animals involves interrelated changes at 3 hierarchical levels: cardiac, valve/hemodynamic, and leaflet ECM/microstructure. Cardiac changes include annular dilation caused by altered strain patterns in the left ventricle due to the HOLE-induced MR; this annular dilation would exacerbate MR. Considering the level of the valve, computer modeling has shown that during normal coaptation, the AML shares the burden of stress with the posterior leaflet. With regurgitation and annular dilation, however, coaptation is reduced, forcing the AML to bear greater stress. Indeed, leaflets under increased stress show increased collagen synthesis. At the level of the ECM and individual valve layers, the collagen Type I-rich fibrosa is responsible for the leaflet’s tensile strength. Based on the greater staining for nonmuscle myosin (a myofibroblastic phenotypic marker) in the fibrosa and the positive correlation between this staining and the average final MR, it appears that the fibrosa is experiencing increased stress related to MR. It also appears that collagen remodeling is occurring with Type I collagen being replaced by flexible reticular Type III collagen. Although this remodeling was likely initiated in reaction to the altered stresses, the altered collagen proportions could reduce the tensile strength of the AML and worsen MR.

Pathological Versus Adaptive Leaflet Remodeling

Although this study provides the first evidence to date that regurgitation alone is sufficient to cause leaflet remodeling, it
is difficult to say whether these changes in the AML should be considered pathological (detrimental) or adaptive (advantageous). From a clinical and pathological perspective, these changes would seem to be detrimental. Certainly, a decrease in the amount of Type I collagen would make the leaflet more prone to prolapse, but it is not clear whether other changes such as increased expression of P4H and similar markers should be considered detrimental. Increased DCN expression has been reported in prolapsed mitral valves, but it is not clear whether the changes in DCN expression were causing the prolapse or attempting to counteract prolapse. It is also important to note that in this study, only a single time point was examined. Therefore, the reduction in collagen may have been a transient event in a process of adaptive remodeling to restore leaflet integrity that would be followed by the production of new collagen (as suggested by the greater expression of P4H). Other findings, however, suggest that this remodeling is “pathological.” Elevated MMP-9 expression has been associated with a number of valve pathologies, including nonrheumatic aortic stenosis. Increases in other MMPs have also been reported in diseased valves, including myxomatous mitral valves and stenotic aortic valves. Myxomatous mitral valves also demonstrate a higher ratio of Type III to Type I collagen, as suggested by the results of this study.

**Potential Mechanism for “MR begets MR”**

This unique animal model created isolated regurgitation to investigate how the valve was affected by low-pressure volume overload and showed that regurgitation alone can initiate leaflet remodeling. Several animal models of chronic MR have been created previously, but these have all involved the confounding effects of ventricular ischemia and remodeling and/or created regurgitation using high-pressure volume overload, which is significantly different than the hemodynamics seen in clinical MR. For instance, the severance of chordae has been shown to alter left ventricular geometry and strain patterns and cause global systolic dysfunction. Many cases of chronic MR involve factors other than altered hemodynamics that contribute to increasing regurgitation. A paradigm often used to account for chronically increasing MR is that ventricular dilation, which frequently accompanies chronic MR, increases ventricular wall stress and impairs left ventricular ejection performance, which then augments regurgitation. Others see MR as principally due to annular dilatation that results in impaired leaflet coaptation. In these views, the MR is “functional,” a result of changes in left ventricular structure and function. However, the findings of the present study suggest that MR itself could be an independent factor contributing to chronically increasing MR through leaflet remodeling.

These results have implications for a number of clinical conditions. In this article, we have shown that a primarily hemodynamic insult results in secondary organic changes with functional consequences. Hemodynamics may similarly affect very different conditions such as myxomatous mitral valves. Leaflets that have undergone organic changes (ie, myxomatous degeneration) may be sensitive to changes in loading that could further deteriorate cardiac function. Likewise, myxomatous valves in which regurgitation has been surgically corrected would experience improved hemodynamics and may undergo positive remodeling. Overall, changes in left ventricular and annular structure and function, organic changes in the mitral valve leaflets, and cardiac and valvular hemodynamic changes are interrelated factors that distinctly influence one another and trigger further cardiac dysfunction.

**Limitations**

Caution should be exercised in extrapolating the results of this ovine study to human hearts. One of the most important limitations was the duration of MR. Although in patients, MR progresses slowly over the course of years, these animals had isolated MR for only 12 weeks. Regardless, significant differences were found even within this short time period. Another limitation was that increasing MR was not detected over the 12-week period. This finding was likely due to the short time course and the decrease in the posterior leaflet’s hole size as it healed (analysis of the posterior leaflet was reported separately). Furthermore, although the changes in HOLE were primarily driven by MR, there were secondary changes in left ventricular dimensions such as annular dilation that could have affected leaflet composition. However, all of the effects on leaflet composition were either directly or indirectly caused by MR. Another limitation was the narrow range of MR in the HOLE animals. A wider range of MR, combined with more specific hemodynamic measurements, would have enabled detection of more direct correlations between hemodynamics and AML compositional changes.

Limitations in the leaflet analysis included the subjective nature of histological grading. To make this process more objective, a grading rubric was used for each characteristic, the grader was blinded to leaflet identity, and all leaflets stained for a given marker were evaluated at the same time. Another limitation was that the surgical and histological analyses were performed at different institutions, so it was not possible to perform materials testing on these tissues or to link material properties directly to matrix changes. In the future, it will be important to analyze the remodeling and material behavior of other parts of the mitral valve, including the fibrous trigones, the chordae, and the other regions of the AML, because loading patterns across the valve are very heterogeneous.

**Conclusion**

Using a novel animal model of isolated MR, this study provides the first evidence that regurgitation alone can result in leaflet remodeling. The HOLE AML showed elevated expression of MMPs and reduced expression of Type I collagen along with greater abundance of P4H (a marker of active collagen synthesis), DCN (a proteoglycan involved in collagen fibrillogenesis), and Type III collagen. Taken together, these changes suggest an increase in matrix degradation and in collagen synthesis, particularly Type III collagen synthesis. Furthermore, greater abundance of elastin in the spongiosa and fibrosa layers of the HOLE AML and higher levels of the elastin-degrading MMP-9 suggest that elastin remodeling is also occurring.
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Disclosures
None.

References

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